



Supporting Information

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Mass Spectrometric Method for Analyzing Metabolites in Yeast with Single Cell Sensitivity

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The medium-term goal of this work is to integrate microfluidic cell sampling, sizing, lysis, and coarse separation of the cell components with detection by mass spectrometry. The key challenge for such combined microfluidics – MS analysis of cellular metabolites is sensitivity. The different strategies that were considered and the optimization steps we took for the one chosen – MALDI-MS – are discussed here.

- **ESI vs. MALDI** mass spectrometry. Biological samples, including compounds from within cells, are easily analyzed by electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. ESI is highly versatile and easily connected on-line with separation techniques, including microfluidic-based separations [S1]. However, the multiply charged ions typically produced in ESI lead to a decrease of sensitivity compared to MALDI (which produces predominantly singly charged ions), because the ion current is distributed over several peaks, and an individual peak of an ESI charge state distribution is more easily lost in the noise. In addition, it is not feasible to record ESI-MS spectra from very small (picoliters-range) volumes of samples; an individual yeast cell has a volume of < 100 fL. Experimentally, nano-ESI mass spectrometry was used to test the sensitivity with our model mixture of metabolites. 40nl of sample solution was injected, corresponding to an amount of 790 amoles of ATP. The ESI spectra clearly showed a lower sensitivity compared to MALDI, even when a greater sample consumption was allowed. For this reason, MALDI was chosen as a detection method. MALDI mass spectrometry has additional advantages in terms of ease of sample preparation, throughput, and tolerance to salts, which is important for analysis of biological samples.

• **On-line vs. off-line MALDI.** In recent years, efforts have been made to develop on-line liquid sample delivery for MALDI, with the aim of coupling MALDI with (continuous) separation methods such as a liquid chromatography. On-line MALDI concepts include direct introduction of a small flow of liquid into the vacuum by a capillary [S2], ionization at the end of an LC column by atmospheric pressure MALDI, “painting” the effluent of a liquid delivery onto a wheel [S3], tape [S4], or a rotating sphere [S5], which moves the sample past a vacuum seal into the ion source of a MALDI instrument, and others. While on-line interfacing of LC with ESI is a natural choice, direct coupling of LC with MALDI is more problematic. Atmospheric pressure MALDI [S6] has a sensitivity that is orders of magnitude below that of vacuum MALDI; moving a MALDI sample past a high vacuum requires constant maintenance as well as construction of a special ion source, which precludes the use of optimized commercial instrumentation; handling liquid samples in vacuum causes notorious problems due to solvent evaporation and freezing. Overall, interfacing a continuous sample delivery, such as a flow from a microchip with MALDI mass spectrometry, being a pulsed method, is not a natural choice. Due to these considerations (including our own experience with on-line MALDI [S7 – S9]), off-line sample preparation / spotting was deemed the better choice than on-line sample delivery. Importantly, off-line sampling for MALDI provides better sensitivity, due to the development of optimized sample preparation procedures and instruments.

• **Negative vs. positive ion mode.** In general, MALDI MS analysis is carried out in positive ion mode [S10], although there is no clear evidence that positive ions are more abundant in general. We suspect that the ion optics for many commercial instruments have been optimized for positive ion mode. In some cases, e.g. the analysis of phosphopeptides or nucleic acids, both positive and negative modes can

obviously be beneficial [S11]. The analysis of cellular metabolites – nucleotides and phosphorylated species – is clearly such a case. Our experiments were therefore carried out in negative ionization mode. The choice of a proper matrix is a critical factor affecting on ionization yield in MALDI. 9-aminoacridine (9-AA) was used for detection in negative mode as a MALDI matrix [S12]. This matrix showed a low degree of fragmentation and cluster formation, and resulted in only minor interference for analysis of small molecules [S12].

- **MALDI vs. DIOS.** There have been efforts to avoid matrix altogether in the analysis of small molecules. One approach, called Desorption Ionization on Silicon (DIOS) was also tested in this work. Standard DIOS was found to be completely unsuitable for the target metabolites. In a second step, commercially available DIOS plates were oxidized in an ozone plasma. A chemical modification of surface was then carried out by silylation with (3-aminopropyl) dimethylethoxysilane and 10-(carbomethoxy)decyl-dimethylchlorosilane. A mixture of model metabolites was dissolved in water and diluted with MeOH (1:1). DIOS-MS experiments were conducted attaching DIOS chips to a modified MALDI target plate with a conductive double adhesive tape. For comparison against MALDI, the metabolite mixture was mixed 1:1 (v:v) with 9-AA (10mg/ml in acetone). All the measurements were performed with a standard MALDI time-of-flight instrument (Axima-CFR, Kratos/Shimadzu, Manchester/UK) in linear negative ion mode. Compared to MALDI with 9-AA, we found a markedly lower sensitivity using the modified DIOS chips. The functionalization of the porous silicon surface did allow the detection of phosphorylated metabolites, but MALDI showed better sensitivity in detection of metabolites by about one order of magnitude (Supporting Figure 1). Despite of fact

that DIOS does not generate any matrix peaks in the low m/z region of the spectrum, use of matrix was found to give much better results in terms of sensitivity.

• **MALDI sample preparation: “dried droplet” vs. thin layer.** Sample preparation in MALDI is directly connected to its ionization efficiency. In general, MALDI gives spot-dependent spectra. In this work, the sample preparation is critically affecting sensitivity. Two methods, “dried droplet” and layered sample preparation were compared to find the better condition for MALDI of metabolites. For the “dried droplet” method, an equal volume of matrix solution and analyte solution were mixed. 1 μ l of this mixture solution was immediately deposited on a sample plate. For the layered preparation, a droplet of analyte (0.5 μ l dispensed with a pipette) was deposited on top of a thin matrix layer, created by fast evaporation of 9-AA dissolved in acetone. Small crystals of the homogeneous, thin matrix layer provide seeds for subsequent co-crystallization with the analyte. When we compared two spots with scanning electron microscopy (SEM), spots produced by the dried droplet method were found to form larger crystals than the layered sample preparation (Supporting Figure 2). The respective MALDI MS spectra are compared in Supporting Figure 3, and the S/N of the metabolites peaks in Supporting Figure 4. These intensities were referenced to the noise level in a peak-free region. The thin layer preparation led to an increase of the analyte peak intensities up to a factor of 2 in the case of Acetyl-CoA, and to a decrease of the chemical background signals in the low molecular weight range (Fig. 3B). The layered preparation gave more reproducible spectra, without big fluctuations of matrix peaks.

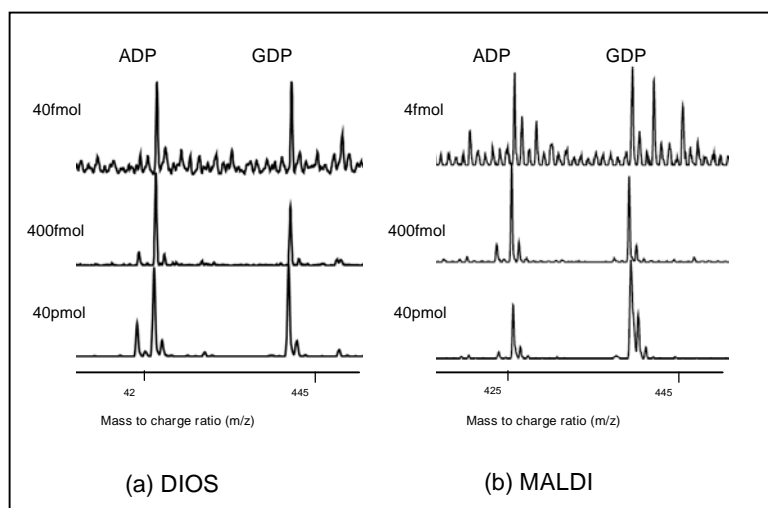
• **Optimum layer thickness.** The next step was to optimize the thickness of the matrix layer for the layered preparation. We used different concentrations of matrix solution to create different layer thicknesses. A lower concentration of matrix, i.e., a

thinner layer, gave a better signal-to-background ratio because the interference with matrix peaks was reduced. However, using a pipette, it is generally difficult to define the layer thickness accurately. For more precise experiments, electrospray deposition of matrix was used. With electrospray deposition the layer thickness can be controlled, because the spot diameter is well-defined. When the pipetting and the electrospraying were compared by SEM, the pipette preparation showed differences in density, size, and shape of the matrix crystallites. The rim of the spot formed a thicker layer than the center in pipette preparation, while electrospraying gave a homogeneous circular spot (Supporting Figure 5). Electrospraying was performed using a small needle with a 152 μm inner diameter. The needle was positioned 3mm above a sample plate, and 2kV was applied. Experiments were carried out using various spraying times (5s, 10s, 20s, and 30s) and a 1 $\mu\text{l}/\text{min}$ flow rate. The spectra resulting from matrix layers deposited for 5 and 10s were better than the ones from 20s and 30s, in agreement with previous findings [S13]. In any case, pipetting or electrospraying showed that the thin matrix layer results in a better sensitivity. Two representative metabolites (PEP and UDP) were compared (Supporting Figure 6). The results clearly show that thinner matrix layers yield increased noise ratios.

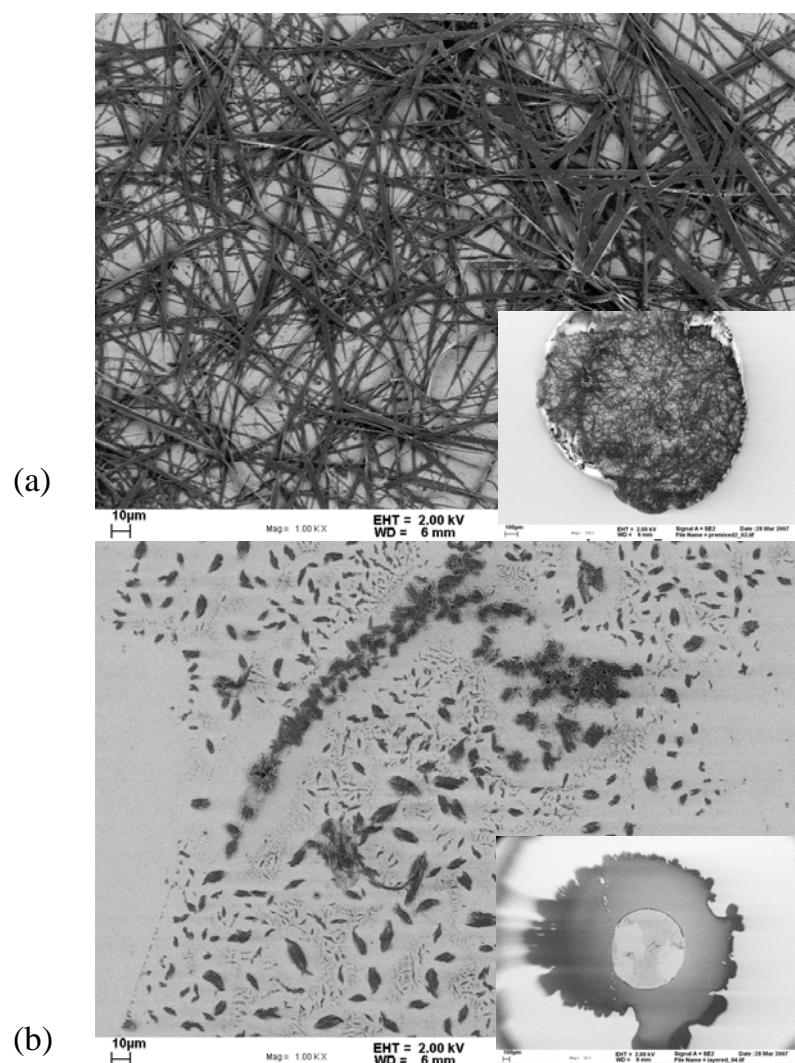
Following the above results, we used MALDI in negative mode to detect metabolites. MALDI samples were prepared off-line using 9-aminoacridine as the matrix. The analyte solution was deposited on top of thin matrix layer using a piezoelectric dispenser to optimize sensitivity in MALDI. While every single optimization step improved the sensitivity by a factor 2 ... 10 only, the combined effect of all optimization allowed us to detect metabolites in the range of tens of amol.

References for Supporting Information

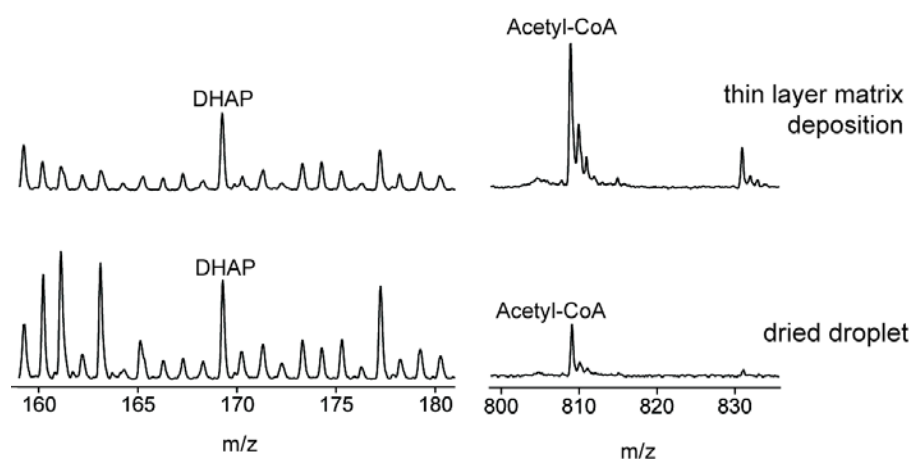
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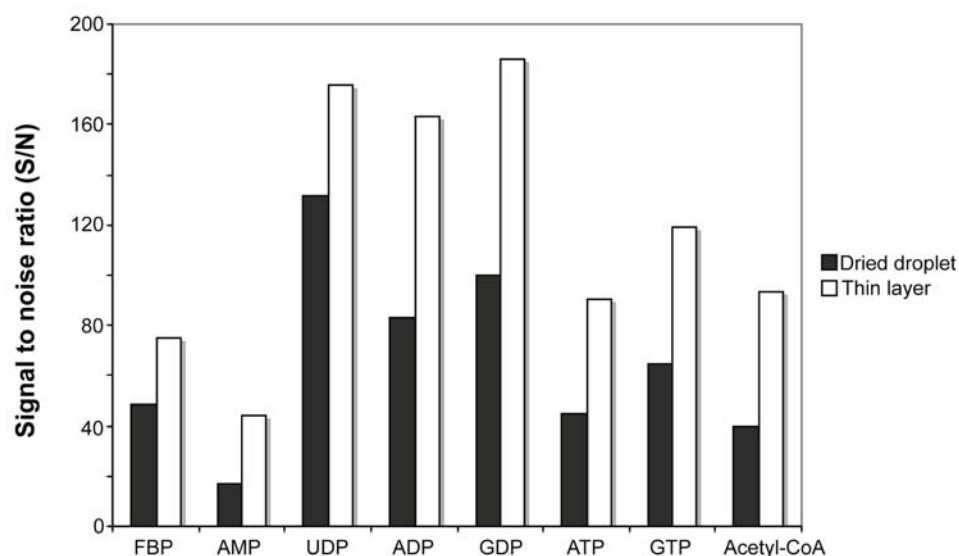
Supporting Figure 1. DIOS with the amino modified surface (a) and MALDI (b) mass spectra obtained for various analyte concentrations. The detection limit of MALDI (top spectrum, 4 fmol) was about one order of better than for DIOS (40 fmol). These spectra were measured using a traditional sample preparation with deposition of 1 μ L of sample in order to establish which of the two techniques is more sensitive for the metabolites studied. MALDI was then used with the microscale preparation in order to reach the single cell sensitivity (see Fig 1 of the main paper).



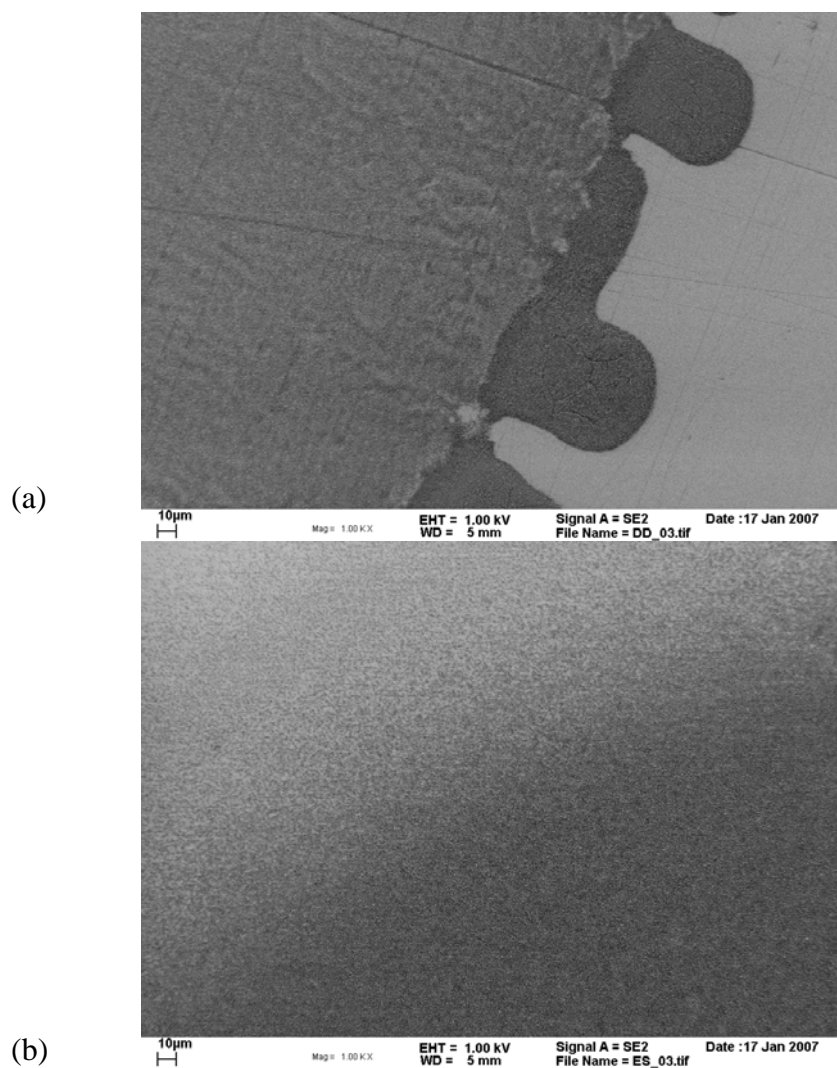
Supporting Figure 2. SEM images of a “dried droplet” sample (a) and a layered sample (b). Each SEM image was taken in the center of the spot. The insets show the full image for each spot.



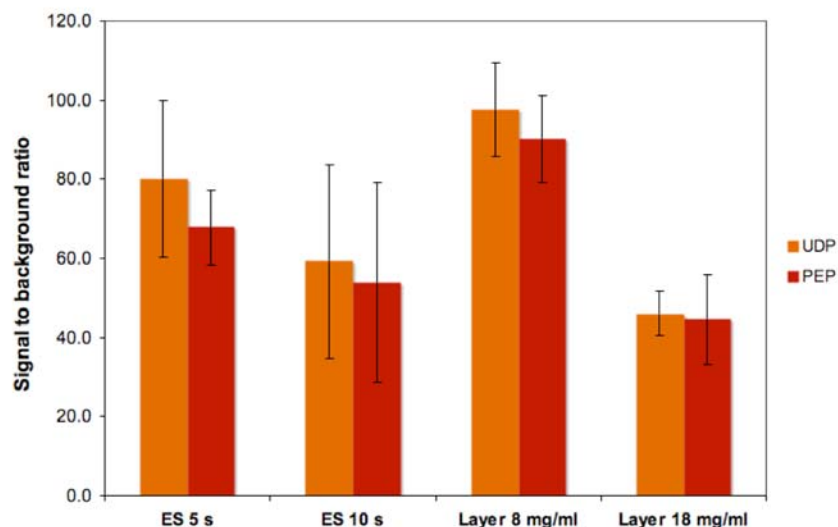
Supporting Figure 3. MALDI MS spectra of a mixture of Dihydroxyacetone phosphate (DHAP) and Acetyl-Coenzyme A recorded using the “dried droplet” (lower trace) and thin layer (upper trace) preparation. The “dried droplet” spot shows stronger interferences from matrix peaks in low mass range.



Supporting Figure 4. The signal-to-noise ratio from Supporting Figure 3, was compared for each metabolite signal for the “dried droplet” and layered sample preparation methods. This graph clearly shows that a better signal-to-noise ratio is obtained for the layered preparation.



Supporting Figure 5. SEM images of the edge of the matrix layer prepared by pipetting (a) and by electro spraying (b). In the pipette preparation, matrix forms smaller and fewer crystals in the center, while many larger crystal formed on the edge of the spot. Electro spraying gave a homogeneous layer with a similar size of crystallites throughout the spot.



Supporting Figure 6. Comparison of the signal-to-noise ratio for UDP and PEP for different matrix thickness in the layered preparation. ES5 and ES10 represents data acquired from a spot prepared by electrospraying for 5s and 10s, respectively. Layer 8mg/ml and layer 18mg/ml represents data from a matrix layer prepared by the pipette preparation using a concentration of 8mg/ml and 18mg/ml, respectively. In both cases the preparations leading to a thinner matrix layer (i.e. ES5s and layer8mg/ml) result in a higher signal to background ratio, with a confidence coefficient of 93% and 99% respectively for the electrospray preparation and thin layer preparation (according to a t-test conducted on the values of the UDP).